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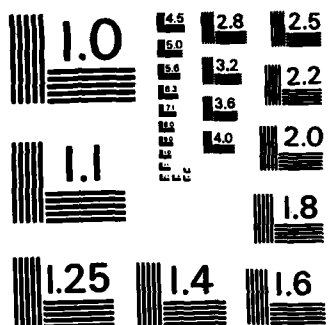
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## THE LUNG SURFACTANT SYSTEM IN ADULT RESPIRATORY DISTRESS SYNDROME

## FINAL PROGRESS REPORT

John U. Balis

August 1980

Sponsored by:

US Army Medical Research and Development Command  
Fort Detrick, Frederick, MD 21701

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University of South Florida  
College of Medicine  
12901 North 30th Street  
Tampa, FL 33612STIC  
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human surfactant. We have already characterized the phospholipid composition of normal adult human surfactant and made considerable progress in the isolation and characterization of lung specific proteins. These proteins are somewhat different than those found in other animal species, although the major peptide subunits appear to be common in most animal species studied. In addition, we have begun to evaluate the phospholipid composition of surfactant isolated from tracheal aspirates of patients with ARDS. The preliminary data are promising with respect to our search for biochemical markers of lung surfactant damage. We have also obtained experimental evidence that ligation of rat cecum is a suitable model for gram negative sepsis and DAD.



## SUMMARY

This research project was designed to characterize the components of the normal human lung surfactant system in order to evaluate the mechanisms, extent and significance of surfactant alterations which may accompany the progression of diffuse alveolar damage (DAD) in patients treated for ARDS. Using purification procedures developed in our laboratory we have been able to demonstrate that surgically excised lung specimens, postmortem lung specimens and tracheal aspirates are suitable sources for the isolation of human surfactant. We have already characterized the phospholipid composition of normal adult human surfactant and made considerable progress in the isolation and characterization of lung specific proteins. These proteins are somewhat different than those found in other animal species, although the major peptide subunits appear to be common in most animal species studied. In addition, we have begun to evaluate the phospholipid composition of surfactant isolated from tracheal aspirates of patients with ARDS. The preliminary data are promising with respect to our search for biochemical markers of lung surfactant damage. We have also obtained experimental evidence that ligation of rat cecum is a suitable model for gram negative sepsis and DAD.

## STATEMENTS

- (1) For the protection of human subjects the investigator has adhered to policies of applicable Federal Law 45CFR46.
- (2) In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal, Resources National Academy of Sciences-National Research Council.

## 1. Studies of Human Surfactant.

This research project is designed to characterize the components of the normal human lung surfactant system and to evaluate the mechanisms, extent and significance of surfactant alterations which may accompany the progression of diffuse alveolar damage in patients treated for ARDS. During the past year we have characterized biochemically "normal" human lung surfactant and begun to investigate the composition of surfactant isolated from tracheal aspirates of intubated patients with or without ARDS.

The method of surfactant purification by centrifugation on discontinuous density gradients was found to be equally effective for both minced lung tissue and tracheal aspirates. As expected, two consecutive density gradient centrifugations were needed to remove the large excess of protein from the surfactant band. To obtain highly purified surfactant, the surfactant fraction collected from the second density gradient centrifugation is dialyzed for 24 hours at 4°C against distilled water and then purified on yet another discontinuous density gradient. Quantitatively, only very small amounts of phospholipid and protein are lost from the surfactant fraction with this step, and the composition of this highly purified material is very consistent from sample to sample.

We have purified surfactant from 15 specimens of grossly and histologically normal portions of surgically removed human lung specimens. Using the method described above, the composition of the surfactant from various human specimens is quite consistent. The phospholipid to protein ratio is  $11.6 \pm 0.4$ . The major phospholipid, phosphatidylcholine (PC) which is  $76.4\% \pm 1.0$  of the total phospholipid, contains  $79.4 \pm 1.4\%$  palmitic acid (16:0),  $2.7 \pm 0.4\%$  myristic acid (14:0), and  $3.7 \pm 0.4\%$  stearic acid (18:0). These saturated fatty acids, thus account for 86% of the total PC fatty acids. The mono-unsaturated fatty acids, palmitoleic (16:1) and oleic (18:1) are present as  $5.6 \pm 0.5\%$  and  $7.5 \pm 1.0$ , respectively. The only other fatty acid present to the extent of more than 1% of the total PC fatty acids is linoleic (18:2),  $1.1 \pm 0.4\%$ . Less than 0.1% of the total PC fatty acid is arachidonic acid (20:4).

Phosphatidylglycerol (PG) is the most abundant phospholipid,  $10.5\% \pm 0.9$  of the total. Phosphatidylinositol, phosphatidylserine, sphingomyelin, phosphatidylethanolamine and an unidentified phospholipid account for the remaining 13% with none more than an average of 4%. These values differ slightly from human amniotic fluid surfactant, in which we have found a wider range of normal values. However, amniotic fluid surfactant was evaluated after only 1 or 2 density gradient centrifugations and further study of highly purified amniotic fluid surfactant using the method described above may yield more consistent values.

Our attempts to measure the molecular species of PC in lung surfactant have yielded only 2 major fractions, one of which has been identified as 16:0, 16:1 PC and the other larger fraction which contains both dipalmitoyl PC (16:0, 16:0) and 16:0, 18:1 PC. In order to quantitate these latter 2 components it is necessary to separate saturated PC from PC with fatty acids containing 1 or more double bonds prior to high performance liquid chromatographic analysis. This has been accomplished by using a cryochromatography procedure. We have separated known standards of several other PCs which we would expect to find in surfactant, based on the fatty acid composition, and have determined by monitoring at 230 nm that we have adequate sensitivity to detect fairly small quantities of these PCs. These results suggest that no other major molecular species of PC is present in normal lung surfactant.

Study of the protein components of purified surfactant was initially hampered by the fact that, unlike the rabbit lung surfactant, delipidated and lyophilized human surfactant could not be dissolved in sodium dodecyl sulfate (SDS) solutions. This problem was overcome by analyzing surfactant samples on SDS-polyacrylamide gels without prior delipidation. To solubilize the surfactant which contains about 12 times more lipid than protein, a 10% SDS solution was used. These studies revealed that the major lung surfactant proteins are high molecular weight proteins (M.W. > 400,000). There are at least 2 protein bands and on some gels a smaller amount of a third band can be seen. Using 2-dimensional slab gel electrophoresis we have demonstrated that all of these high molecular weight proteins, on reduction of disulfide bonds with dithiothreitol, yield 35,000 dalton peptides. Similar proteins are also present in amniotic fluid surfactant, but the ratios of the high molecular weight components are somewhat different. The importance of this finding is not yet known. Since all the high molecular weight proteins are composed of the same peptide subunits, the various proteins may reflect some difference in polymerization related to lipid binding properties. In highly purified adult human surfactant these peptides are about 80% of the total protein stained with coomassie blue on SDS-PAGE. The remainder of the proteins is largely albumin with only small amounts of other peptides. Both the high molecular weight bands and the peptide derived from them after reduction of disulfide bonds stain with PAS, indicating that they are glycoproteins, in agreement with previous reports of the 36,000 dalton peptide of human amniotic fluid (Biochim. Biophys. Acta 537: 329, 1978). When the surfactant band is collected from a density gradient tube in several small fractions, the fractions near the bottom of the band contain relatively more protein than do those at the top of the band, where phospholipid to protein may reach a ratio of 24/1. Analysis of the top versus the bottom fractions of the surfactant bands revealed only one difference in phospholipid and protein composition. In undelipidated surfactant fractions with a high phospholipid to protein ratio the presence of a small amount of a low molecular weight (less than 20,000) protein was observed. Although the amount of this protein is small compared to the high molecular weight component, its presence is of interest in light of the recent report of lipophilic peptides in porcine surfactant (Biochem. J. 183:731, 1979).

We have also purified surfactant from a normal lung obtained at autopsy. Both the phospholipid and protein components of the surfactant were the same as that found in the surgically removed specimens. Therefore, we are confident that reliable data can be obtained by evaluation of postmortem lung samples from patients with or without ARDS.

One or more serial tracheal aspirates have been obtained from 13 intubated patients. Our attempts to obtain surfactant from these aspirates were successful in 6 cases. The amount of surfactant recovered from these samples limits the number of studies which may be carried out. Since we have previously found in hyaline membrane disease of the newborn that the fatty acid composition of the PC differs greatly from that of normal controls, we first examined this parameter. Two normal controls (intubated patients without ARDS) had essentially normal PC fatty acid compositions. Three specimens were obtained early in the course of ARDS and two of these were entirely normal. In the third, the amount of palmitic acid was about 10% less than the normal value, while the 18-carbon unsaturated fatty acids were higher than normal. Arachidonic acid, which is present in normal surfactant as less than 0.1% of the total PC fatty acids, was found to be 1.2%. We also evaluated two aspirates from 1 patient recovering from severe ARDS. In this patient a lung biopsy performed seven days



prior to obtaining the tracheal aspirate demonstrated diffuse alveolar damage with hyaline membrane formation. In addition to low 16:0 and elevated 18 and 20-carbon unsaturated fatty acids, palmitoleic acid was also elevated, a change similar that seen in the recovery phase of neonatal hyaline membrane disease.

The results obtained during the past year clearly demonstrated that surgically excised lung specimens, postmortem lung specimens and tracheal aspirates are suitable sources for the isolation of human surfactant. We have already characterized the phospholipid composition of normal adult human surfactant and made considerable progress in the isolation and characterization of lung specific proteins. These proteins are somewhat different than those found in other animal species, although the major peptide subunits appear to be common in most animal species studied. In addition, we have begun to evaluate the phospholipid composition of surfactant isolated from tracheal aspirates of patients with ARDS. The preliminary data are promising with respect to our search for biochemical markers of lung surfactant damage.

## 2. Ligation of Rat Cecum as a Model for DAD.

We have recently performed a series of experiments which have enabled us to develop a reliable rat model for DAD. In this model, the standard procedure involves ligation of the distal 2 cm of the rat cecum under aseptic conditions. This procedure results in gangrene of the ligated cecal segment with abscess formation and peritonitis. Survival in the above model is largely dependent upon the size of the ligated cecal segment. Following total cecal ligation, the rats uniformly die within 24 hours. With the standard procedure, the survival rate is about 60% and 40% at 24 and 120 hours respectively. In rats killed at 24, 48, 72 and 120 hours gram negative bacteria (E. coli, Bacteroides fragilis or Klebsiella) were consistently isolated from the peritoneum, blood, lung, liver and kidney. Characteristic hematologic changes were observed in blood samples obtained at 24 hours and 48 hours after either cecal ligation or sham operation. These changes included persistent mild leukopenia and thrombocytopenia elevation of fibrinogen level, prolongation of PT and PTT, and decrease in the level of coagulation factors VII and X at 24 hours (Tables I and II). These findings indicate that cecal ligation and associated peritonitis and gram negative sepsis lead to low grade disseminated intravascular coagulation (DIC), as it is often the case in patients with "chronic" gram negative sepsis. Histologic and electron microscopic evaluation of lung tissue revealed progressive congestive atelectasis, margination of leukocytes and platelet aggregates in the microcirculation of the lung and other organs, and prominent endothelial damage in association with interstitial pulmonary edema. Moreover, sequential alveolar reactions were consistently observed, and these included damage of the type I epithelium, increase accumulation of alveolar macrophages and, at 72-120 hours, striking increase in the number of type II cells as well as infiltration of the septa with lymphoid and histiocytic cells. The above histopathologic changes are similar to those seen in DAD or interstitial pneumonitis. Therefore, ligation of rat cecum appears to be an excellent animal model for gram negative sepsis and ARDS.

#### Recent Publications, Resulting From This Research

1. Shelley, S.A., Paciga, J.E., Richman, A.V. and Balis, J.U.: Characterization of the Human Surfactant System. Fed. Proc. 38:1372, 1979.
2. Mason, R.G., Mohammad, S.F., Paterson, J.F., Balis, J.U.: Endothelial Injury in Umbilical Cord Veins Perfused with Suspensions of E. Coli. Circulatory Shock 6:170, 1979.
3. Balis, J.U., Shelley, S.A., Paterson, J.F., Paciga, J.E., Gerber, L.I.: Mechanism of Congestive Atelectasis in Endotoxin Shock. Circulatory Shock 6:187, 1979.
4. Paciga, J.E., Shelley, S.A. and Balis, J.U.: Secretory IgA - a major protein component of rabbit lung surfactant. Biochim Biophys Acta 631:487-494, 1980.
5. Richman, A.V., Gerber, L.I. and Balis, J.U.: Peritubular capillaries - a major target site for endotoxin induced vascular injury in the primate kidney. Lab. Invest. 43:327-332, 1980.
6. Richman, A.V., Okulski, E.G. and Balis, J.U.: New concepts in the pathogenesis of acute tubular necrosis associated with sepsis. Annals of Clinical and Laboratory Science 11(3):211-219, 1981.
7. Lotz, M.J., Fareed, J. and Balis, J.U.: Role of splenic red pulp in endotoxin-induced disseminated intravascular coagulation. Lab. Invest. 45(5):469-476, 1981.
8. Shelley, S.A., Balis, J.U., Paciga, J.E., Espinoza, C.G. and Richman, A.V.: Biochemical composition of adult human lung surfactant. Lung 160:195-206, 1982.

#### Statement

No equipment was acquired through the University of South Florida; however, the Beckman Analytical Ultracentrifuge, Model E, was transferred by Certificate of Property Transfer executed November 16, 1978.

There were no supplies remaining by the end of the extended grant period.

T A B L E I

## CAECAL LIGATION MODEL - HEMATOLOGIC DATA

	LEUKOCYTES ( $\times 10^3/\text{cu.mm.}$ )	PLATELETS ( $\times 10^3/\text{cu.mm.}$ )	FIBRINOGEN ( $\text{mg./dl.}$ )	PTT ( $\text{sec.}$ )	PT ( $\text{sec.}$ )
Normal control	5.3 $\pm$ 0.5 (6)	642 $\pm$ 31 (6)	208 $\pm$ 6 (5)	18.8 $\pm$ 0.5 (5)	10.6 $\pm$ 0.1 (5)
Sham Operated Control (24 hrs)	3.8 $\pm$ 0.7 (8)	628 $\pm$ 29 (6)	402 $\pm$ 34 <sup>a</sup> (6)	19.7 $\pm$ 0.6 <sup>a</sup> (6)	11.2 $\pm$ 0.1 <sup>a</sup> (6)
Sham Operated Control (48 hrs.)	4.5 $\pm$ 0.6 (4)	667 $\pm$ 34 (5)	267 $\pm$ 19 (5)	18.3 $\pm$ 0.6 (5)	11.0 $\pm$ 0.2 (5)
Experimental (24 hrs)	2.5 $\pm$ 0.4 <sup>a,b</sup>	431 $\pm$ 23 <sup>a,b</sup>	588 $\pm$ 11 <sup>a,b</sup>	28.1 $\pm$ 0.4 <sup>a,b</sup>	14.2 $\pm$ 0.4 <sup>a,b</sup>
Experimental (48 hrs)	2.4 $\pm$ 0.3 <sup>a,b</sup> (9)	465 $\pm$ 24 <sup>a,b</sup> (9)	681 $\pm$ 36 <sup>a,b</sup> (9)	34.4 $\pm$ 3 <sup>a,b</sup> (9)	12.5 $\pm$ 0.5 <sup>a,b</sup> (9)

<sup>a</sup>Value is significantly different from normal control

<sup>b</sup>Value is significantly different from sham operated control

T A B L E    II

## CAECAL LIGATION MODEL - COAGULATION FACTORS

	<u>XII</u>	<u>VIII</u>	<u>X</u>	<u>VII</u>
Normal Control	>100 (7)	78+7 (7)	>100 (7)	88+7 (7)
Sham Operated Control (24 hrs)	86+7 (6)	88+6 (6)	>100 (6)	77+7 (6)
Sham Operated Control (48 hrs)	95+3 (5)	>100 (5)	>100 (5)	70+9 (5)
Experimental (24 hrs)	90+5 (9)	92+3 (9)	25+3 <sup>a, b</sup> (9)	18+3 <sup>a, b</sup> (9)
Experimental (48 hrs)	67+7 (9)	63+8 (9)	>100 (9)	58+11 <sup>a</sup> (9)

<sup>a</sup>Value is significantly different from normal control

<sup>b</sup>Value is significantly different from sham operated control

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